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Improvement in lipase-catalyzed methanolysis of triacylglycerols for biodiesel production using a solvent engineering method

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ABSTRACT

A solvent engineering strategy was applied to the lipase-catalyzed methanolysis of triacylglycerols for biodiesel production. The effect of different pure organic solvents and co-solvent mixtures on the methanolysis was compared. The substrate conversions in the co-solvent mixtures were all higher than those of the corresponding pure organic solvents. Further study showed that addition of co-solvent decreased the values of $|\log P_{\text{interface}} - \log P_{\text{substrate}}|$ and thus led to a faster reaction. The more the values of $|\log P_{\text{interface}} - \log P_{\text{substrate}}|$ decreased, the faster the reaction proceeded and the higher the conversion attained. Different co-solvent ratio was further investigated. The co-solvent mixture of 25% *t*-pentanol:75% isooctane (v/v) was optimal, with which both the negative effects caused by excessive methanol and by-product glycerol could be eliminated. There was no obvious loss in lipase activity even after being repeatedly used for 60 cycles (720 h) with this co-solvent mixture as reaction medium. Other lipases and lipase combinations can also catalyze methanolysis in this co-solvent mixture. Furthermore, other vegetable oils were also explored for biodiesel production in this co-solvent mixture and it had been found that this co-solvent mixture media has extensive applicability.

1. Introduction

According to the US Standard Specification for Biodiesel (ASTM 6751), it is defined as a fuel comprised of mono-alkyl esters of long chain fatty acids derived from vegetable oils or animal fats. In this context, it can be used in diesel engines and heating systems [1]. Biodiesel has clear benefits in comparison with diesel fuel. Since biodiesel comes from renewable sources, it does not contribute to new carbon dioxide emission—one of the factors responsible of the greenhouse effect—as diesel from petroleum does [2]. The other main characteristic of this fuel is the almost total absence of sulphur and the low production of soot particulate after combustion [3–5]. In this sense, numerous scientific articles dealing with biodiesel production have been published. More recently, the demand for biodiesel has increased even more due to petroleum price rises and the development of government measures like The EU Directive 2003/30/EC on the promotion of the use of biofuels to accelerate the use of alternative fuels in the transportation sector.

Different processes are currently available to achieve transesterification of triacylglycerols for the production of biodiesel, which include chemical or enzyme catalysis or supercritical alcohol treatment [6–11]. Chemical processes give high conversion of triacylglycerols to their corresponding esters but have drawbacks such as being energy intensive, difficulty in removing the glycerol, and the need for removal of alkaline catalyst from the product and treatment of alkaline wastewater. Use of biocatalysts (lipases) in transesterification of triacylglycerols for biodiesel production addresses these problems and offers an environmentally more attractive option to the conventional processes [12–15]. Although enzymatic approaches have become more and more attractive, they have not been realized in industrialization mainly due to the relatively high price of lipase and its short operational life caused by the negative effects of excessive methanol and by-product glycerol [6,16–19].

It has been demonstrated that more than 1/2 molar equivalent methanol are insoluble in vegetable oils and the immobilized lipases are easily inactivated by contacting with insoluble methanol existing as droplets in the oils [19]. Stepwise addition of methanol [15,19] or using some hydrophobic solvent such as *n*-hexane or petroleum ether as reaction media [14,20,21] have been proposed to reduce the negative effect of methanol on lipase activity. By-product glycerol is hydrophilic and insoluble in the oils, so it is

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easily adsorbed onto the surface of the immobilized lipase also leading to the negative effect on lipase activity and operational stability [21]. Several methods have also been proposed to eliminate the negative effect caused by glycerol: addition of silica gel into the reaction system to absorb the glycerol [22] or washing the lipase with some organic solvents periodically to remove glycerol [21,23,24]. We can find all the proposed methods to solve the negative effects of methanol and glycerol will increase the operational complexity.

The enzyme activity in organic media is often correlated with the solvent hydrophobicity ($\log P$) with the highest activities found at high $\log P$ (>2) values [25–28]. Further, the stability of the enzyme is usually higher in the more hydrophobic solvents. These hydrophobic organic solvents such as *n*-hexane and petroleum ether have also been tried as reaction medium for biodiesel production [20,21,29]. However, methanol and glycerol have poor solubility in these relatively hydrophobic solvents, so the negative effects on lipase activity and stability caused by methanol and glycerol cannot be eliminated and lipase still exhibits poor stability in such reaction media [23]. From the above introduction, we could deduce that a solvent, which could dissolve the hydrophobic oil, hydrophilic methanol and glycerol, might be an appropriate reaction medium. Just as the studies of Li et al. [30], Degn et al. [31] and Castillo et al. [32], in this work, we also applied a solvent engineering method in the search for appropriate reaction medium for lipase-catalyzed methanolysis of triacylglycerols for biodiesel production and found a co-solvent mixture, which could dissolve both methanol and by-product glycerol, so the negative effects caused by them could be eliminated totally.

2. Materials and method

2.1. Materials

Novozym435 (lipase B from *Candida antarctica*, a nonspecific lipase immobilized on a macroporous acrylic resin with a specific activity 10,000 PLU/g and water content 1–2% (w/w)), Lipozyme TL IM (lipase from *Thermomyces lanuginosus*, immobilized on the particles of silica gel with a specific activity 175 IUN/g and water content 5% (w/w)) and Lipozyme RM IM (lipase from *Mucor miehei*, immobilized on a macroporous anionic resin with a specific activity 5–6 BAUN/g and water content 2–3% (w/w)) were bought from Novo Nordisk Bioindustrials Inc. Methyl esters of palmitic acid, stearic acid, oleic acid, linoleic acid, linolenic acid, erucic acid, ricinoleic acid and heptadecanoic acid were from Sigma and were chromatographically pure. Pistacia chinensis Bunge oil, *Jatropha curcas* L. oil, soybean oil, rapeseed oil, corn oil, peanut oil, castor oil, olive oil, cottonseed oil,sunflower oil were purchased locally. 1,4-Dioxane, *t*-butanol and *t*-pentanol were obtained from commercial supplier (Shanghai Chemical Company, China). All other chemicals and reagents were obtained commercially and were of analytical grade. All the organic solvents were treated with molecular sieve 3 Å for several days before using.

2.2. General procedure for lipase-catalyzed methanolysis

Under standard conditions, reactions were performed as batch process in 5 mL of pure organic solvents or co-solvent mixtures in 25 mL screw-caped glass vials containing 0.5 mmol vegetable oil, 1.5 mmol methanol and 10% of lipase (based on oil weight). The reactions were carried out at 45 °C and 150 rpm in a shaker fitted with a thermostat. These conditions were used except when otherwise stated in the text. Samples (6 μL) taken from the reaction mixture at specified times were mixed with 7.5 μL of 20 mM heptadecanoic acid methyl ester (served as internal standard) and 136.5 μL of *n*-heptane, and were analyzed by gas chromatography.

2.3. GC analysis of the samples

Samples prepared as described above were analyzed by injecting 1 μL of *n*-heptane solution and internal standard into an Agilent 6890 gas chromatography, equipped with a HP-5 capillary column(5% phenyl methyl siloxane capillary, 30.0 m × 320 μm × 0.25 μm nomimal). The column temperature was kept at 180 °C for 1 min, heated to 300 °C at 10 °C/min, and then maintained for 2 min. The temperatures of the injector and detector were set at 260 and 280 °C, respectively. Under these conditions, the retention times for palmitic acid methyl ester, heptadecanoic acid methyl ester, oleic acid methyl ester, linoleic acid methyl ester, linolenic acid methyl ester, stearic acid methyl ester, erucic acid methyl ester and ricinoleic acid methyl ester were 5.122, 5.916, 6.499, 6.474, 6.563, 6.702, 9.697 and 8.121 min, respectively. All samples were measured in triplicate, with reproducibility always within 3%. The conversion was defined as the concentration ratio of transformed oil to initial oil × 100.

2.4. IR analysis

Infrared (IR) spectrum of the viscous sample obtained by washing the Novozym 435 with water after transesterification was determined on Nicolet Magna-IR550.

2.5. Determination of free glycerol content in the reaction mixture

The free glycerol in the reaction mixture was measured according to the method of Bondioli and Della Bella [33]. Into a 10 mL test tube, the right amount of sample, dissolve in 4 mL of hexane and add 4 mL of working solvent (mix equal volumes of distilled water and 95% ethanol). Shake the sample vigorously for 5 min. Centrifuge for 15 min at 2000 rpm. Remove the main part of the upper layer. Transfer exactly 0.5 mL of the lower layer into a 10 mL test tube. Add 1.5 mL of working solvent. Add 1.2 mL of a 10 mM sodium periodate solution and shake for 30 s. After that, add 1.2 mL of a 0.2 M acetylacetone solution and put in a water bath thermostated at 70 °C for 1 min, stirring manually. After the reaction time, the sample must be immediately cooled by immersing the tube in a beaker containing tap water. The samples are finally read in a spectrophotometer set in double beam mode at 410 nm, against a blank sample prepared in the same way as the samples, after addition of 2 mL of working solvent to the test tube.

2.6. Stability of Novozym435

The stability of Novozym435 during batch transesterification reactions was investigated. In the co-solvent mixture system, the Novozym435 was reused directly without any treatment after 12 h reaction in each cycle and the methanol was added one time. In the pure isooctane system, the Novozym435 was reused directly without any treatment after 24 h reaction in each cycle and the methanol was added three times.

2.7. Solvent hydrophobicity

The solvent hydrophobicities ($\log P$ values is defined as the logarithm of the partition coefficient in an octanol–water two-phase system) used in the present work were taken from Laane et al. [26] or Carrea et al. [34]. The hydrophobicity of co-solvent mixtures can be calculated from the Eq. (1) [35].

$$\log P_{\text{mix}} = x_1 \log P_1 + x_2 \log P_2 \quad (1)$$

Table 1
Lipase-catalyzed transesterification of *Jatropha curcas* L. seed oil with methanol in pure solvents and co-solvents with different log *P* values

Pure solvents	Conversion (%)[a]	log *P*	Co-solvents[b]	Conversion (%)	log *P*
Isooctane	8.8	4.7	*n*-hexane:dioxane	9.59	2.23
n-Heptane	5.5	4.0	*n*-Heptane:dioxane	9.79	2.47
n-Hexane	5.7	3.5	Isooctane:dioxane	12.34	2.8
Tetrachloromethane	1.7	3.0	*n*-Hexane:*t*-Butanol	35.86	2.81
Toluene	2.9	2.5	*n*-Heptane:*t*-butanol	38.94	3.11
Benzene	2.2	2.0	Isooctane:*t*-Butanol	45.9	3.51
t-Pentanol	28.8	1.4	*n*-Hexane:*t*-pentanol	34.23	3.02
t-Butanol	22.9	0.80	*n*-Heptane:*t*-pentanol	38.81	3.35
Tetrahydrofuran	0.9	0.49	Isooctane:*t*-pentanol	52.13	3.8
Acetonitrile	16.6	−0.33			
Dioxane	8.0	−1.1			

Reaction conditions: molar ratio of methanol/oil 3:1; 10% Novozym435 based on the oil weight; temperature 45 °C; 150 rpm; reaction time 4 h.
[a] Transesterification activity was expressed as the conversion of substrate.
[b] Mixture of solvent (4:1,v/v).

where x_1 and x_2 are the mole fractions of components 1 and 2, and $\log P_1$ and $\log P_2$ are the pure component values.

3. Results and discussion

3.1. Effect of reaction medium

One of the most troublesome bottleneck in lipase-catalyzed methanolysis of triacylglycerols for biodiesel production is the poor solubility of methanol in most organic solvents, such as isooctane, *n*-heptane and *n*-hexane [20,21,29]. We investigated the lipase-catalyzed transesterification of *J. curcas* L. seed oil with methanol in different pure nonpolar solvents. As shown in Table 1, low substrate conversion was observed in the solvents with high hydrophobicity. This result can therefore be ascribed to the excessive insoluble methanol in the reaction medium deactivating the lipase gravely. In fact, only polar organic solvents, such as *t*-butanol, have been used for dissolving methanol [36,37]. Therefore, we also investigated the lipase-catalyzed transesterification of *J. curcas* L. seed oil with methanol in different pure polar solvents. High substrate conversions were found in *t*-butanol, *t*-pentanol, acetonitrile and 1,4-dioxane (Table 1). Generally solvents with a log *P* value below 2 have been considered unsuitable as media for enzyme catalysis [26]. In the present work, high enzyme activity was observed in solvents with a log *P* value below 2, and significant enzyme activity could even be detected in acetonitrile and 1,4-dioxane which both have very low log *P* values. This result can be explained that these solvents can dissolve methanol and thus overcome its negative effect on lipase to some degree. However, polar organic solvents usually strip the essential water off the lipase molecules and then inactivate the biocatalyst and thus influence the stability of lipase during repeated use, which greatly limits the application of enzymatic procedures in this area [38]. As an alternative, co-solvent mixtures which were composed of a high log *P* solvent such as isooctane and a low log *P* solvent such as 1,4-dioxane were tried. Because acetonitrile is not soluble in isooctane, *n*-heptane and *n*-hexane, their co-solvent mixtures did not study in this work. The results were shown in Table 1. The substrate conversions in the co-solvent mixtures were all higher than those of the corresponding pure solvents. This result demonstrated that higher lipase tolerance to methanol and stability could be achieved in co-solvent mixtures. Laane reported that there was some influence of the log *P* value of the reaction medium on the reaction rate. They also found that the lower the $|\log P_{\text{interface}} - \log P_{\text{substrate}}|$ or the higher the $|\log P_{\text{continuous phase}} - \log P_{\text{substrate}}|$ was, the easier/faster the reaction proceeded [26,35]. Thus, the log *P* of reactants was proposed here to analyze the improved lipase tolerance to methanol. Since the reactants include triglycerides and methanol, therefore the $\log P_{\text{substrate}}$ was calculated according to the semiempirical Eq. (1). Here x_1 and x_2 stand for the molar ratio of methanol and triglycerides, respectively. The interface of the lipase was supposed to be a layer of water for pure solvent system or a uniform layer of mixtures of water and dioxane or *t*-butanol or *t*-pentanol for co-solvent mixture system. Adopting the isooctane co-solvent mixture system as an example, Table 2 gave the log *P* value of reactant and the values of $|\log P_{\text{interface}} - \log P_{\text{substrate}}|$ were also calculated accordingly. It could be seen that addition of co-solvent decreased the values of $|\log P_{\text{interface}} - \log P_{\text{substrate}}|$ and thus led to a faster reaction. The more the values of $|\log P_{\text{interface}} - \log P_{\text{substrate}}|$ decreased, the faster the reaction proceeded and the higher the conversion attained. This indicated that higher lipase tolerance to methanol was realized by addition of co-solvent.

3.2. Effect of the co-solvent ratio

As stated above, the addition of co-solvent could improve the lipase tolerance to methanol and stability. In order to find an appropriate co-solvent mixture for lipase-catalyzed methanolysis of triacylglycerols for biodiesel production, we investigated the effects of different co-solvent ratio. As shown in Table 3, the conversion of oil in *t*-pentanol co-solvent mixture > that in *t*-butanol co-solvent mixture > that in 1,4-dioxane co-solvent mixture. The

Table 2
Effect of co-solvent addition on the substrate conversion and $|\log P_i - \log P_s|$

Solvents	$\log P_i$[a]	$\log P_s$[b]	$\lvert\log P_i - \log P_s\rvert$	Conversion (%)[c]
Isooctane	−1.44	5.795	7.235	8.8
Isooctane:dioxane (4:1)[d]	−1.27	5.795	7.065	12.34
Isooctane:*t*-butanol (4:1)	−0.32	5.795	6.115	45.9
Isooctane:*t*-pentanol (4:1)	−0.02	5.795	5.815	52.13

Reaction conditions: molar ratio of methanol/ *Jatropha curcas* L. seed oil 3:1; 10% Novozym435 based on the oil weight; temperature 45 °C; 150 rpm; reaction time 4 h.
[a] $\log P_i = \log P_{\text{interface}}$.
[b] $\log P_s = \log P_{\text{substrate}}$.
[c] Transesterification activity was expressed as the conversion of substrate.
[d] Mixture of solvent (v/v).

Table 3
Lipase-catalyzed transesterification of *Jatropha curcas* L. seed oil with methanol in different co-solvent mixtures with varied ratio

Co-solvent mixtures (v/v)	Conversion (%)	Co-solvent mixtures (v/v)	Conversion (%)	Co-solvent mixtures (v/v)	Conversion (%)
Dioxane:5% *n*-hexane	8.27	*t*-Butanol:20% *n*-hexane	26.69	*t*-Pentanol:10% *n*-hexane	29.30
Dioxane:10% *n*-hexane	8.54	*t*-Butanol:30% *n*-hexane	27.47	*t*-Pentanol:20% *n*-hexane	29.36
Dioxane:20% *n*-hexane	8.60	*t*-Butanol:40% *n*-hexane	28.50	*t*-Pentanol:30% *n*-hexane	30.18
Dioxane:30% *n*-hexane	8.79	*t*-Butanol:50% *n*-hexane	29.47	*t*-Pentanol:40% *n*-hexane	32.76
Dioxane:40% *n*-hexane	8.90	*t*-Butanol:60% *n*-hexane	31.70	*t*-Pentanol:50% *n*-hexane	33.23
Dioxane:50% *n*-hexane	9.07	*t*-Butanol:70% *n*-hexane	33.51	*t*-Pentanol:60% *n*-hexane	35.80
Dioxane:60% *n*-hexane	9.18	*t*-Butanol:80% *n*-hexane	35.86	*t*-Pentanol:70% *n*-hexane	37.76
Dioxane:70% *n*-hexane	9.34	*t*-Butanol:90% *n*-hexane	24.58	*t*-Pentanol:80% *n*-hexane	34.23
Dioxane:80% *n*-hexane	9.79			*t*-Pentanol:90% *n*-hexane	24.75
Dioxane:90% *n*-hexane	7.05	*t*-Butanol:5% *n*-heptane	23.20		
		t-Butanol:10% *n*-heptane	24.74	*t*-Pentanol:5% *n*-Heptane	31.78
Dioxane:5% *n*-heptane	8.36	*t*-Butanol:20% *n*-heptane	25.46	*t*-Pentanol:10% *n*-heptane	32.57
Dioxane:10% *n*-heptane	8.48	*t*-Butanol:30% *n*-heptane	26.67	*t*-Pentanol:20% *n*-heptane	33.58
Dioxane:20% *n*-heptane	8.51	*t*-Butanol:40% *n*-heptane	27.67	*t*-Pentanol:30% *n*-heptane	33.95
Dioxane:30% *n*-heptane	8.84	*t*-Butanol:50% *n*-heptane	28.02	*t*-Pentanol:40% *n*-heptane	35.47
Dioxane:40% *n*-heptane	9.16	*t*-Butanol:60% *n*-heptane	35.50	*t*-Pentanol:50% *n*-heptane	36.47
Dioxane:50% *n*-heptane	10.04	*t*-Butanol:70% *n*-heptane	38.10	*t*-Pentanol:60% *n*-heptane	38.12
Dioxane:60% *n*-heptane	11.08	*t*-Butanol:80% *n*-heptane	38.94	*t*-Pentanol:70% *n*-heptane	44.79
Dioxane:70% *n*-heptane	11.20	*t*-Butanol:90% *n*-heptane	27.30	*t*-Pentanol:80% *n*-heptane	38.81
Dioxane:80% *n*-heptane	9.59			*t*-Pentanol:90% *n*-heptane	26.45
Dioxane:90% *n*-heptane	8.30	*t*-Butanol: 5% isooctane	26.40		
		t-Butanol:10% isooctane	27.60	*t*-Pentanol: 5% isooctane	31.13
Dioxane: 5% isooctane	8.13	*t*-Butanol:20% isooctane	27.90	*t*-Pentanol:10% isooctane	32.28
Dioxane:10% isooctane	8.24	*t*-Butanol:30% isooctane	29.30	*t*-Pentanol:20% isooctane	36.53
Dioxane:20% isooctane	8.55	*t*-Butanol:40% isooctane	31.60	*t*-Pentanol:30% isooctane	37.56
Dioxane:30% isooctane	8.76	*t*-Butanol:50% isooctane	36.28	*t*-Pentanol:40% isooctane	41.73
Dioxane:40% isooctane	8.88	*t*-Butanol:60% isooctane	38.13	*t*-Pentanol:50% isooctane	44.93
Dioxane:50% isooctane	10.20	*t*-Butanol:65% isooctane	40.36	*t*-Pentanol:60% isooctane	50.14
Dioxane:60% isooctane	12.74	*t*-Butanol:70% isooctane	42.30	*t*-Pentanol:65% isooctane	55.78
Dioxane:70% isooctane	15.85	*t*-Butanol:75% isooctane	43.28	*t*-Pentanol:70% isooctane	58.12
Dioxane:80% isooctane	12.34	*t*-Butanol:80% isooctane	45.90	t-Pentanol:75% isooctane	61.05
Dioxane:90% isooctane	11.55	*t*-Butanol:85% isooctane	44.95	*t*-Pentanol:80% isooctane	52.13
		t-Butanol:90% isooctane	40.13	*t*-Pentanol:85% isooctane	49.94
t-Butanol:5% *n*-hexane	23.67			*t*-Pentanol:90% isooctane	41.15
t-Butanol:10% *n*-hexane	24.64	*t*-Pentanol:5% *n*-hexane	29.21		

Reaction conditions: molar ratio of methanol/oil 3:1; 10% Novozym435 based on the oil weight; temperature 45 °C; 150 rpm; reaction time 4 h. Transesterification activity was expressed as the conversion of substrate.

highest conversion was found in the *t*-pentanol co-solvent mixture with the most suitable composition of 25% *t*-pentanol:75% isooctane (v/v). This result indicates that a mixture of a solvent (*t*-pentanol) in which methanol has a high solubility with a solvent (isooctane) in which the lipase is stable is optimal for lipase-catalyzed synthesis of biodiesel. To the best of our knowledge, this is the first report on lipase-catalyzed synthesis of biodiesel in co-solvent mixture, which improves the conventional lipase-catalyzed synthesis method.

It has been suggested that enzymatic activity for lipase-catalyzed reactions increases with increasing values of the hydrophobicity of the solvent in which the reaction takes place [26]. Therefore, the log *P* values of co-solvent mixtures were used to analyze the changing trend of conversion in different co-solvent ratio. All of the three co-solvents used in this study have lower values of log *P* (see Table 1) than *n*-hexane, *n*-heptane and isooctane so that $\log P_{mix}$ decreases with increasing amounts of co-solvent. Therefore, the transesterification activity (conversion) is expected to decrease with increasing amounts of co-solvent. A plot of transesterification activity (conversion) to $\log P_{mix}$ is shown in Fig. 1. For each co-solvent mixture, there is a consistent trend of increasing conversion with increasing $\log P_{mix}$ up to the point where the solubility limit of the methanol affect the lipase activity. However, mixtures with different co-solvents having the same $\log P_{mix}$ value do not result in the same conversion. For example, at a $\log P_{mix}$ value of 1.44, the conversion changed from 12.74 with dioxane as the co-solvent to 27.47 with *t*-butanol as the co-solvent (Table 3 and Fig. 1). Thus, for the co-solvent mixture system, the observation that increasing $\log P_{mix}$ correlates with increasing enzymatic activity appears to be true when considering a particular co-solvent system, but it does not hold exactly when comparing mixtures with different co-solvents. It should be recognized that (Eq. (1)) does not take into account, in an explicit way, solution non-idealities in the solvent mixtures. The log *P* of a pure component is a description of partitioning of that component between water and a reference organic solvent while the log *P* of a two-component mixture, as defined by Eq. (1) is simply an average of the log *P* values of the individual components. As a result, $\log P_{mix}$ does not take into account specific activity coefficient effects so it can only be used for a qualitative correlation of enzymatic activity.

It is also of interest to note that when the curves in Fig. 1 are extrapolated to the log *P* value of pure *n*-hexane, *n*-heptane and isooctane, the conversions show significant differences. Thus, it appears that even small additions of more polar co-solvent to a highly hydrophobic solvent may dramatically alter the enzymatic activity. A qualitatively similar effect had been reported by Almarsson and Klibanov for the protease subtilisin Carlsberg [39].

3.3. Effect of water addition on the methanolysis in the optimal co-solvent mixture system

The water content is known to be a crucial parameter while using enzymes in non-aqueous media. It is considered more logical to talk in terms of water activity rather than % (w/w) water content [40,41]. However, for a process like biodiesel preparation where the ultimate objective is to work at large scale, it makes better sense to optimize the process simply in terms of addition of different amounts of water. This is so since control of water activity

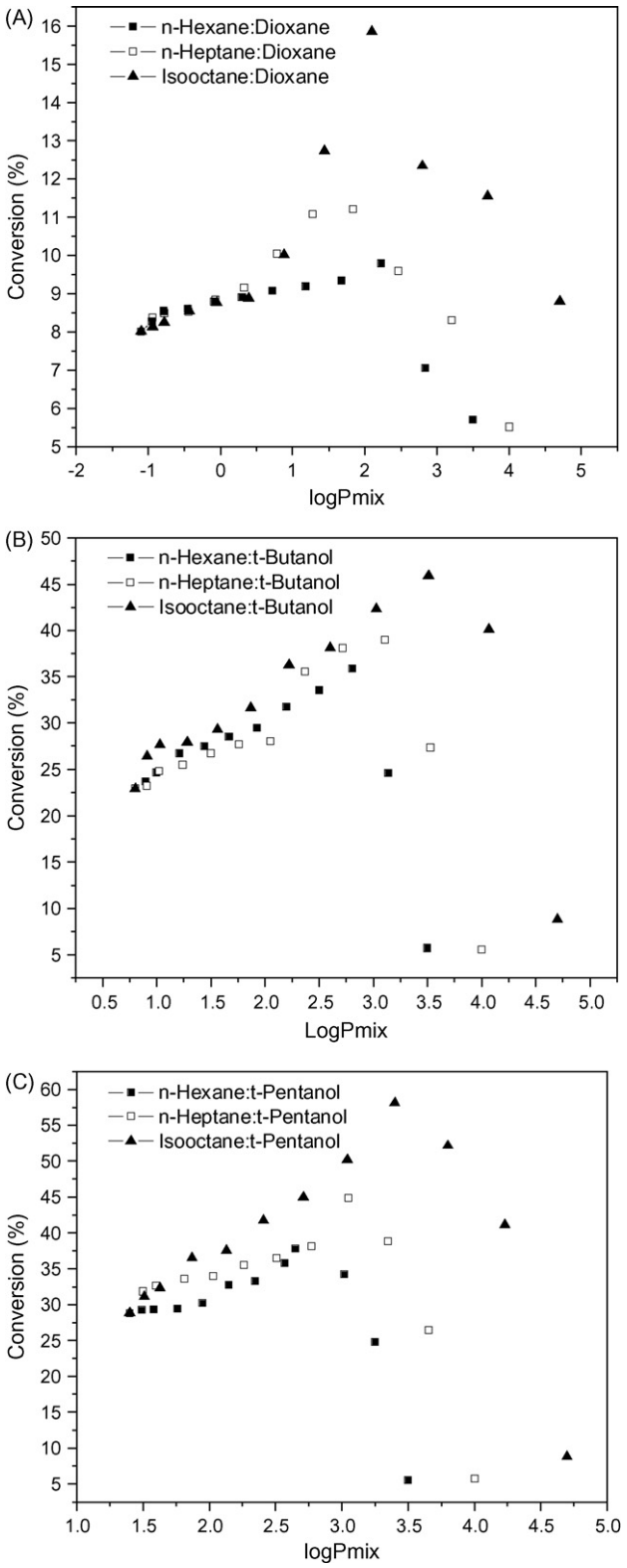

Fig. 1. Substrate conversion plotted as a function of $\log P_{mix}$ in the co-solvent mixtures. (A) Dioxane as co-solvent; (B) *t*-butanol as co-solvent and (C) *t*-pentanol as co-solvent. $\log P_{mix}$ was calculated from Eq. (1). Reaction condition: molar ratio of methanol/*Jatropha curcas* L. seed oil 3:1; 10% Novozym435 based on the oil weight; temperature 45 °C; 150 rpm; reaction time 4 h.

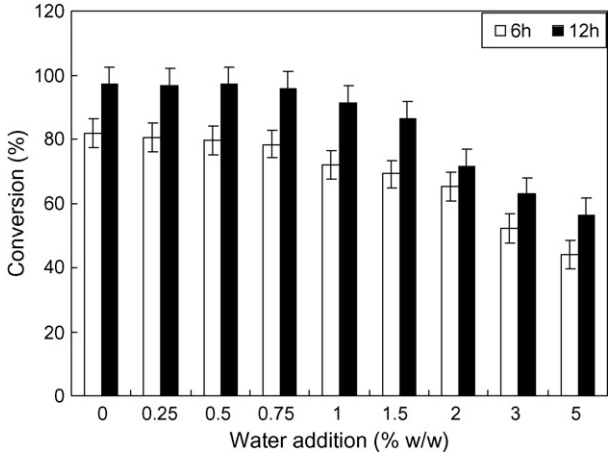

Fig. 2. Effect of water addition on the methanolysis of *Jatropha curcas* L. seed oil in the optimal co-solvent mixture. Reaction conditions: methanol/oil molar ratio 5:1; 7.5% Novozym435 based on the oil weight; temperature 45 °C; 150 rpm. The water addition was based on the weight of oil.

is slightly more complicated in design and may not be convenient at large scale. In non-aqueous systems, there is a minimum and optimum water content required for providing enough conformational flexibility. As water content increases, hydrolysis starts competing with transesterification as the equilibrium shifts towards hydrolysis [42,43]. An additional feature of this process is that fatty acids produced by hydrolysis would again get converted to fatty acid methyl esters. However, this esterification reaction is also favored by low water conditions. That is, in this equilibrium reaction as well hydrolysis of fatty acid methyl esters would be favored over esterification as the water content rises [44]. Also, esterification would produce water molecules, the water content thereby would rise and start further favoring fatty acid methyl esters hydrolysis. So we investigated the effect of water addition on the methanolysis in the optimal co-solvent mixture (25% *t*-pentanol:75% isooctane). As the Fig. 2 showed, when the amount of added water increased from 0 to 0.75 wt%, the conversion scarcely changed. However, when it increased from 0.75 to 5 wt%, the conversion decreased gradually. Therefore, a water addition lower than 0.75% (w/w) on the base of oil weight would be optimum. We selected not to add water to the reaction system in all our experiments.

3.4. Capability of lipase tolerance to methanol in the co-solvent mixture system

Different from conventional lipase-catalyzed methanolysis in solvent-free system or hydrophobic organic solvent system where more than 1/2 molar equivalent methanol are insoluble and thus inactivate the immobilized lipases [19], the toxicity of methanol on lipase activity can be eliminated in the co-solvent mixture system. In order to investigate the capability of lipase tolerance to methanol in the co-solvent mixture system, we chose the optimal co-solvent mixture system (25% *t*-pentanol:75% isooctane) to study the effect of molar ratio of methanol/oil on the methanolysis. As can be seen from Fig. 3, the conversion was enhanced with the increase of methanol concentration until the methanol/oil molar ratio of 5:1. This explained that the added methanol dissolved fully in the co-solvent mixture system and thus did not deactivate the lipase. When the molar ratios were more than 5:1, their conversions were lower than that of molar ratio of 5:1 during the initial 6 h, then attained about the same. This could be due to one-step addition of excessive methanol and some of them could not dissolve perfectly and thus poisoned the lipase to some degree. As the reaction pro-

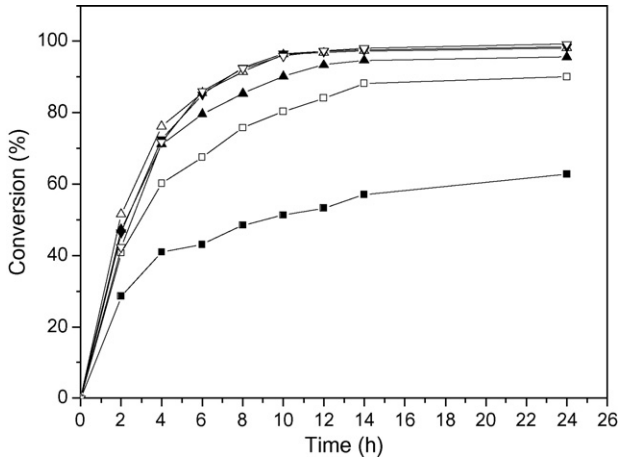

Fig. 3. Capability of lipase tolerance to methanol in the co-solvent mixture system. Reaction conditions: 10% Novozym435 based on the oil weight; temperature 45 °C; 150 rpm. Methanol/*Jatropha curcas* L. seed oil molar ratio: (■) 2:1, (□) 3:1, (▲) 4:1, (□) 5:1, (▼) 6:1, (□) 9:1.

longed, the effect was removed with the consumption of methanol. We can conclude that the capability of lipase tolerance to methanol increase markedly in the co-solvent mixture system.

3.5. Operational stability of Novozym435 in the co-solvent mixture system

One of the most important characteristics of an immobilized enzyme is its stability and reusability over an extended period of time. From the viewpoint of process economics, the more the batch of an enzyme used is, the more economical the process is. An optimal solvent system for lipase-catalyzed methanolysis should not only increase the capability of lipase tolerance to methanol but also not impair the stability of lipase during repeated use. The operational stability of Novozym435 was compared in the co-solvent mixture (25% *t*-pentanol:75% isooctane) system and pure isooctane system. As shown in Fig. 4, the novozym435 lost activity rapidly during repeated use in the pure isooctane system and the conversion closed to zero after five batch reactions

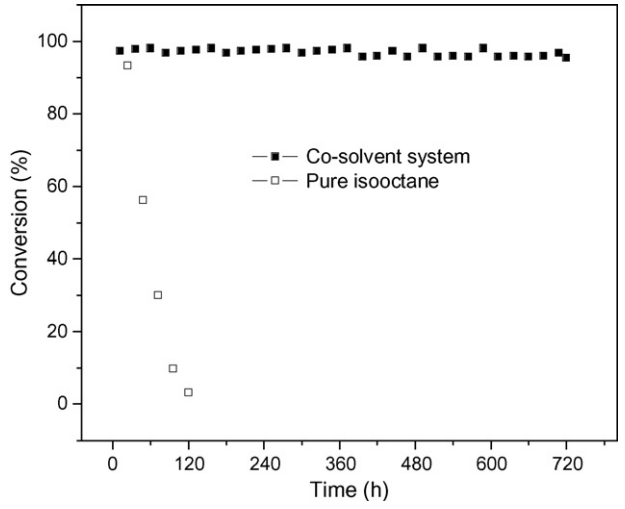

Fig. 4. Operational stability of Novozym435 during batch reaction. Reaction conditions: methanol/*Jatropha curcas* L. seed oil molar ratio of 5:1; 7.5% Novozym435 based on the oil weight; temperature 45 °C; 150 rpm. The methanol added one time (0 h) in co-solvent mixture system and three times (0, 5, and 10 h) in pure isooctane system.

(120 h). However, there was no obvious loss in conversion even after Novozym435 being reused for 60 cycles (720 h). The particles of Novozym435 tended to adhere to each other to form small spheres in the pure isooctane system. However, they dispersed in the co-solvent mixture system uniformly. We filtered the Novozym435 from the pure isooctane system after reaction, washed it with considerable isooctane, then washed with superpure water, filtered again and collected the filtrate for rotary evaporation, viscous sample obtained for IR analysis, the infrared spectrum was showed in Fig. 5, as showed, the viscous sample was 94.62% of similarity to glycerol. We also analyzed the by-product glycerol in the reaction mixture and found that there was theoretic yield of free glycerol existing in the co-solvent mixture system, but only 30% of theoretic yield of free glycerol existed in the pure isooctane system. These results demonstrated that the by-product glycerol could dissolve in the co-solvent mixture system which contributed a lot to the markedly improved lipase stability. However, in the pure isooctane system (traditional enzymatic medium), much glycerol had been adsorbed onto the surface of the immobilized lipase which led to the quite short operational life of the lipases [21–24].

3.6. Other lipases and lipase combinations catalyzed methanolysis in the co-solvent mixture system

It has been reported that both Lipozyme TL IM and Lipozyme RM IM also had high catalytic activity for methanolysis [15,21,45]. So we investigated if they can also be used as biocatalyst for methanolysis of *J. curcas* L. seed oil in this co-solvent mixture (25% *t*-pentanol:75% isooctane). As shown in Table 4, the conversions of both lipases were only about 78% after 24 h reaction and obviously lower than that of Novozym435. This may be because Lipozyme TL IM and Lipozyme RM IM are known as lipases with 1,3-positional specificity and theoretically the highest conversion should be only 67%. Therefore acyl transfer had been thought to occur during the methanolysis, which resulted in the conversion of 78% could be obtained [46]. Furthermore, just as the results of Noureddini's study [43], the different lipase sources, different immobilized methods and carriers may also take some effects on the methanolysis.

Lipozyme TL IM and Lipozyme RM IM are 1, 3-positional specific lipases but with a lower price. Novozym435 is a nonspecific lipase but with a higher price. Considering the cost of lipases and their catalyzed specificity, combined use of these three lipases was

Table 4
Other lipases and lipase combinations catalyzed methanolysis of *Jatropha curcas* L. seed oil in the optimal co-solvent mixture

Lipase	Conversion (%)					
	0 h	2 h	6 h	10 h	12 h	24 h
7.5% A[a]	0	40.5	81.7	91.6	97.3	97.9
7.5% B[b]	0	29	49.5	67.4	73.6	76.5
7.5% C[c]	0	27.5	51.3	68.8	74.5	78.3
1% A + 6.5% B	0	35.6	78.2	87.7	92.5	95.8
2.5% A +5% B	0	39.6	80.2	90.3	96.8	97.7
5% A + 2.5% B	0	41.2	81.4	92.3	96.2	97.4
1% A + 6.5% C	0	32.6	76.9	84.3	93.6	96
2.5% A + 5% C	0	38.6	77.2	87.3	95.4	96.8
5% A + 2.5% C	0	40.1	79.4	89.8	96.2	97.8
1% C + 6.5% B	0	27.4	49.2	65.3	71.8	75.9
2.5% C + 5% B	0	30.6	45.9	62.7	69.4	76
5% C + 2.5% B	0	30.1	50.4	67.3	73.7	76.4

Reaction conditions: methanol/oil molar ratio of 5:1; temperature 45 °C; 150 rpm.
[a] (A) Novozym435.
[b] (B) Lipozyme TL IM.
[c] (C) Lipozyme RM IM.

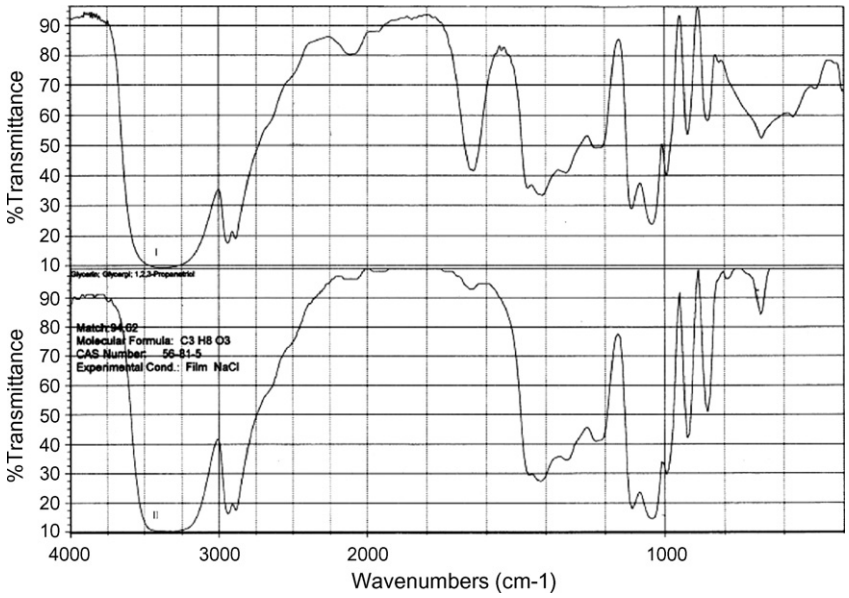

Fig. 5. IR spectrum of viscous sample obtained by washing Novozym435 with superpure water after reaction in pure isooctane system (I) and glycerol (II).

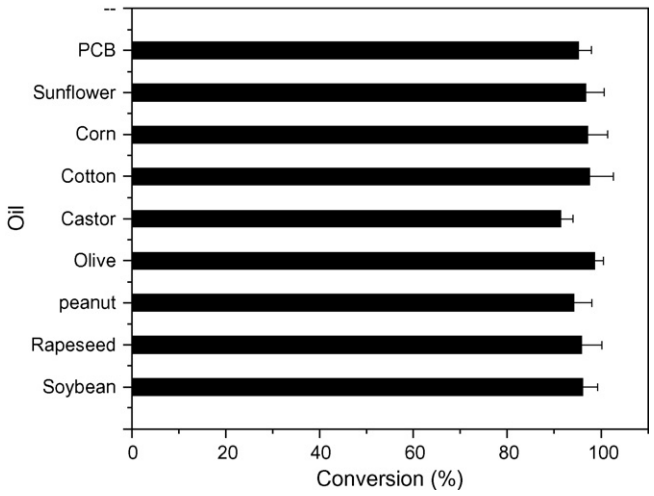

Fig. 6. Novozym435-catalyzed methanolysis of other vegetable oils in the co-solvent mixture. Reaction conditions: methanol/oil molar ratio 5:1; 7.5% Novozym435 based on the oil weight; temperature 45 °C; 150 rpm; reaction time 12 h. PCB: Pistacia chinensis Bunge seed oil.

explored further. As shown in Fig. 5, when 1% Novozym435 was added into 6.5% Lipozyme TL IM or Lipozyme RM IM, the conversion increased remarkably and achieved about the same as that of 7.5% Novozym435 used singly. Therefore, combined use of lipases can cut down the cost of lipase.

3.7. Novozym435-catalyzed methanolysis of other vegetable oils in the co-solvent mixture system

In order to explore if the co-solvent mixture (25% *t*-Pentanol:75% isooctane) has extensive applicability, we investigated the effect of Novozym435-catalyzed methanolysis of other vegetable oils in this co-solvent mixture. As can be seen from the Fig. 6, all the vegetable oils tested attained very high conversion. This shows that this co-solvent mixture can be used as a prospective media for methanolysis of triacylglycerols in biodiesel production.

4. Conclusions

Solvent engineering method was applied to the lipase-catalyzed methanolysis of triacylglycerols for biodiesel production in this work. A co-solvent mixture, 25% *t*-pentanol:75% isooctane (v/v), was optimal and successfully used as the reaction medium for lipase-catalyzed methanolysis for biodiesel production. From the results of this work it can be concluded that the use of this co-solvent mixture in the enzymatic biodiesel production has the following advantages: (a) both the negative effects caused by excessive methanol and by-product glycerol can be eliminated completely; (b) high reaction rates and conversion can be obtained; (c) no catalyst regeneration steps are needed for lipase reuse; and (d) the operational stability of the catalyst is high. In a word, the co-solvent mixture is a very prospective media for methanolysis of triacylglycerols in biodiesel production.

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References

[1] F. Staat, E. Vallet, Chem. Ind. 21 (1994) 863–865.
[2] A. Salis, M. Pinna, M. Monduzzi, V. Solinas, J. Biotechnol. 119 (2005) 291–299.
[3] X. Lang, A.K. Dalai, N.N. Bakhshi, Bioresour. Technol. 80 (2001) 53–62.
[4] G. Antolin, F.V. Tinaut, Y. Briceno, Bioresour. Technol. 83 (2002) 111–114.
[5] G. Vicente, M. Martinez, J. Aracil, Bioresour. Technol. 92 (2004) 297–305.
[6] H. Fukuda, A. Kondo, H. Noda, J. Biosci. Bioeng. 92 (5) (2001) 405–416.
[7] D. Kusdiana, S. Saka, Bioresour. Technol. 91 (3) (2004) 289–295.
[8] F. Ma, M.A. Hanna, Bioresour. Technol. 70 (1) (1999) 1–15.
[9] S. Shah, A. Sharma, M.N. Gupta, Anal. Biochem. 351 (2) (2006) 207–213.
[10] Y. Warabi, D. Kusdiana, S. Saka, Bioresour. Technol. 91 (3) (2004) 283–287.
[11] Y. Zhang, M.A. Dube, D.D. McLean, M. Kates, Bioresour. Technol. 89 (1) (2003) 1–16.
[12] M. Iso, B. Chen, M. Eguchi, T. Kudo, S. Shrestha, J. Mol. Catal. B: Enzym. 16 (1) (2001) 53–58.
[13] O. Kose, M. Tuter, A.H. Aksoy, Bioresour. Technol. 83 (2) (2002) 125–129.
[14] L.A. Nelson, T.A. Foglia, W.N. Marmer, J. Am. Oil. Chem. Soc. 73 (8) (1996) 1191–1195.
[15] Y. Shimada, Y. Watanabe, A. Sugihara, Y. Tominaga, J. Mol. Catal. B: Enzym. 17 (3–5) (2002) 133–142.
[16] W. Du, Y.Y. Xu, D.H. Liu, Z. Jing, J. Mol. Catal. B: Enzym. 30 (3-4) (2004) 125–129.
[17] Y.Y. Xu, W. Du, D.H. Liu, J. Mol. Catal. B: Enzym. 32 (5–6) (2005) 241–245.

[18] M. Kaieda, T. Samukawaa, A. Kondoa, H. Fukudaet, J. Biosci. Bioeng. 91 (2001) 12–15.
[19] Y. Shimada, Y. Watanabe, T. Samukawa, J. Am. Oil Chem. Soc. 76 (1999) 789–793.
[20] P.V. Lara, E.Y. Park, Enzyme Microb. Technol. 34 (2004) 270–277.
[21] M.M. Soumanou, U.T. Bornscheuer, Enzyme Microb. Technol. 33 (2003) 97–103.
[22] D.E. Stevenson, R.A. Stanley, R.H. Furneaux, Enzyme Microb. Technol. 16 (6) (1994) 478–484.
[23] V. Dossat, D. Combes, A. Marty, Enzyme Microb. Technol. 25 (3–5) (1999) 194–200.
[24] E.Z. Su, M.J. Zhang, J.G. Zhang, J.F. Gao, D.Z. Wei, Bichem. Eng. J. 36 (2007) 167–173.
[25] A. Ducret, M. Trani, R. Lortie, Enzyme Microb. Technol. 22 (1998) 212–216.
[26] C. Laane, S. Boeren, K. Vos, C. Veeger, Biotechnol. Bioeng. 30 (1987) 81–87.
[27] T.J. Van, R. Stevens, W. Veldhuizen, J. Jongejan, J. Duine, Biotechnol. Bioeng. 47 (1995) 71–81.
[28] R.H. Valivety, P.J. Halling, A. Macrae, Biochem. Biophys. Acta 1118 (1992) 218–222.
[29] H. Ghamguia, M. Karra-Chaabouni, Y. Gargouri, Enzyme Microb. Technol. 35 (4) (2004) 355–363.
[30] X.F. Li, M.H. Zong, H. Wu, W.Y. Lou, J. Biotechnol. 124 (2006) 552–560.
[31] P. Degn, W. Zimmermann, Biotechnol. Bioeng. 74 (6) (2001) 483–491.
[32] E. Castillo, F. Pezzotti, A. Navarro, A. Lopez-Munguia, J. Biotechnol. 102 (2003) 251–259.
[33] P. Bondioli, L. Della Bella, Eur. J. Lipid Sci. Technol. 107 (2005) 153–157.
[34] G. Carrea, G. Ottolina, S. Riva, Trends Biotechnol. 13 (1995) 63–70.
[35] M.H. Hilhorst, R. Spruijt, C. Laane, C. Veeger, Eur. J. Biochem. 144 (1984) 459–466.
[36] L. Wang, W. Du, D.H. Liu, L.L. Li, N.M. Dai, J. Mol. Catal. B: Enzym. 43 (2006) 29–32.
[37] D. Royon, M. Daz, G. Ellenrieder, S. Locatelli, Bioresour. Technol. 98 (2007) 648–653.
[38] M.Y. Lee, J.S. Dordick, Curr. Opin. Biotechnol. 13 (2002) 376–384.
[39] O. Almarsson, A.M. Klibanov, Biotechnol. Bioeng. 49 (1996) 87–92.
[40] P.J. Halling, Enzyme Microb. Technol. 16 (3) (1994) 178–206.
[41] P.J. Halling, Enzymic conversions in organic and other low-water media, in: K. Drauz, H. Waldmann (Eds.), Enzymes Catalysis in Organic Synthesis, vol. I, Wiley VCH, Weinheim, 2002, p. 259.
[42] S. Shah, S. Sharma, M.N. Gupta, Energy Fuel 18 (2004) 154–159.
[43] H. Noureddini, X. Gao, R.S. Philkana, Bioresour. Technol. 96 (2005) 769–777.
[44] T. Yamane, Y. Kojima, T. Ichiryu, M. Nagata, S. Shimizu, Biotechnol. Bioeng. 34 (1989) 838–843.
[45] Y.Y. Xu, W. Du, J. Zeng, D.H. Liu, Biocatal. Biotransform. 22 (2004) 45–48.
[46] W. Du, Y.Y. Xu, D.H. Liu, Z.B. Li, J. Mol. Catal. B: Enzym. 37 (2005) 68–71.